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Neurobiology

NUB1 Suppresses the Formation of Lewy Body-Like Inclusions by Proteasomal Degradation of Synphilin-1

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NUB1 is a potent down-regulator of the ubiquitin-like protein NEDD8, because it targets NEDD8 to the proteasome for proteolytic degradation. From results in this study, we found that NUB1 physically interacts with synphilin-1 through its NEDD8-binding site, implying that NUB1 also targets synphilin-1 to the proteasome for degradation. Synphilin-1 is a major component of inclusion bodies found in the brains of patients with neurodegenerative α-synucleinopathies, including Parkinson's disease. In this study, we immunostained sections of brains from patients with Parkinson's disease and other α-synucleinopathies and demonstrated that NUB1, as well as synphilin-1, accumulates in the inclusion bodies. To define the role of NUB1 in the formation of these inclusion bodies, we performed a co-transfection assay using cultured HEK293 cells. This assay showed that NUB1 suppresses the formation of synphilin-1-positive inclusions. Further, biochemical assays revealed that NUB1 overexpression leads to the proteasomal degradation of synphilin-1. These results and our previous observations suggest that NUB1 indeed targets synphilin-1 to the proteasome for its efficient degradation, which, because of the resultant reduction in synphilin-1, suppresses the formation of synphilin-1-positive inclusions. ***(Am J Pathol 2006, 169:553–565; DOI: 10.2353/ajpath.2006.051067)***

NEDD8 is a ubiquitin-like protein that conjugates to a large number of target proteins in a manner analogous to ubiquitination.[1] These target proteins include cullin family members, the von Hippel-Lindau tumor suppressor gene product, and p53.[2–4] Because NEDD8 conjugation modifies the function of target proteins, the conjugation system appears to regulate many important biological events.[3–5]

Recently, we identified a novel down-regulator of the NEDD8 conjugation system, NUB1.[6] NUB1 is a NEDD8-interacting protein composed of 601 amino acid residues with a calculated molecular mass of 69.1 kd. It possesses a ubiquitin-like (UBL) domain at the N-terminal region and two ubiquitin-associated (UBA) domains at the C-terminal region. In a biochemical analysis, we found that NUB1 recruits NEDD8 and its conjugates to the proteasome for degradation, making NUB1 a down-regulator in the NEDD8 conjugation system.[6,7] Most recently, to elucidate the function of NUB1, we performed a yeast two-hybrid screening using NUB1 as bait and isolated the cDNA of synphilin-1 from a human cDNA library. Synphilin-1, a 919-amino acid protein, is an α-synuclein-interacting protein whose function is currently unknown. It is predominantly expressed in neurons, localized in the cytoplasm and presynaptic nerve terminals, and is thought to be involved in the pathogenesis of Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA), collectively referred to as α-synucleinopathies.[8–11]

PD is a common neurodegenerative disorder that is characterized by the loss of midbrain dopamine neurons[12] and the presence of Lewy bodies (LBs), proteinaceous cytoplasmic inclusions that contain ubiquitin, NEDD8, α-synuclein, synphilin-1, and parkin.[9,10,13,14] Importantly, the mutations of genes encoding α-synuclein, synphilin-1, and parkin have been linked to familial forms

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of PD, indicating that functional derangements of these proteins have prominent roles in the pathogenesis of PD.[15–18]

In the study described here, we demonstrated the interaction between NUB1 and synphilin-1 and investigated the possible role of NUB1 in the formation of inclusion bodies in the neurodegenerative disorders related to synphilin-1, such as PD and other α-synucleinopathies.

Materials and Methods

Cell Culture

Human embryonic kidney HEK293 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics.

Antibodies

Mouse anti-HA antibody 16B12 was purchased from Covance (Richmond, CA). Mouse anti-RH antibody (specific for the amino acid sequence RGSHHHH) was purchased from Qiagen (Santa Clara, CA). Mouse anti-ubiquitin antibody 1B3 was purchased from MBL (Nagoya, Japan). GST-12, a mouse monoclonal antibody specific for glutathione *S*-transferase (GST), was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-synphilin-1 antibody (AB5450) and goat anti-synphilin-1 antibody (AB5838) were purchased from Chemicon (Temecula, CA) and used for Western blotting and tissue staining, respectively. Mouse anti-phosphorylated α-synuclein antibody no. 64 (specific for α-synuclein phosphorylated at Ser129) was purchased from Wako (Osaka, Japan).[19] Rabbit anti-actin antibody was purchased from Sigma (St. Louis, MO). Mouse monoclonal antibodies against the Rpt5 subunit of PA700 and the α-subunits of the 20S proteasome were purchased from Affiniti Research Products Ltd. (Mamhead, Exeter, UK). Mouse anti-FLAG antibody (M5) was purchased from Sigma. Rabbit anti-NEDD8 antibody was generated by immunization with a GST-fusion protein of human NEDD8, followed by affinity purification.[7] Rabbit anti-human NUB1 antiserum was generated by immunization with a GST-fusion protein of NUB1 corresponding to amino acids 432 to 601, followed by affinity purification.[6] Briefly, the IgG component was purified from the serum using a protein G-Sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ). The IgG fraction was then passed over the GST column twice to remove antibodies to GST. The flow-through was subjected to the GST-NUB1 column for affinity purification. The purified anti-NUB1 antibody (100 μg/ml) was diluted and used.

Construction of Prokaryotic Expression Plasmids

To express GST fusion proteins in *Escherichia coli* BL21 cells, cDNAs of HHR23B and NUB1 were subcloned into the pGEX-2TK plasmid (Amersham Pharmacia Biotech). To express RH-tagged synphilin-1 in *E. coli* BL21 cells, the cDNA was subcloned into the pTrcHis plasmid (Invitrogen, Carlsbad, CA).

Construction of Mammalian Expression Plasmids and Transfection

To experimentally form cytoplasmic inclusions, synphilin-1 was co-expressed with the NAC portion of α-synuclein in HEK293 cells. For this co-expression, we generated a plasmid to simultaneously express both EGFP-fused synphilin-1 and FLAG-tagged NAC. We constructed a pcDNA3 plasmid (Invitrogen) in which there are two sites for protein expression, a multiple cloning site (MCS) and a site for the neomycin-resistant gene (Neo). The Neo cDNA was first replaced with the cDNA of FLAG-NAC to generate a plasmid pNAC. Furthermore, the cDNA of synphilin-1 fused with EGFP at its C-terminus (Sph1-EGFP) was either inserted into the MCS of pcDNA3 to generate pSph1-EGFP or inserted into the MCS of pNAC to generate pNAC-Sph1 for the simultaneous expression of both synphilin-1-EGFP and FLAG-NAC. As a control vector, the cDNA of EGFP alone was either inserted into the MCS of pcDNA3 to generate pEGFP or inserted into the MCS of pNAC to generate pNAC-EGFP for the simultaneous expression of both EGFP and FLAG-NAC. These plasmids were transfected into HEK293 cells using FuGENE6 (BD Biosciences, San Jose, CA).

Yeast Two-Hybrid Assay for Screening of the Human cDNA Library

To identify NUB1-interacting proteins, human testis cDNA library (Clontech, Mountain View, CA) was screened using the yeast two-hybrid assay system (Clontech) as described previously.[20]

Yeast Two-Hybrid Assay for the Interaction of Synphilin-1 with Truncated NUB1

We performed a yeast two-hybrid assay to assess the interaction of synphilin-1 with truncated NUB1. First, by using a polymerase chain reaction (PCR) with appropriate primers, we prepared cDNAs of human synphilin-1[8] and the truncated NUB1 as described previously.[21] The yeast Matchmaker Two-Hybrid System 3 (Clontech) was used to examine the *in vivo* interaction of synphilin-1 with these mutants. To do so, the cDNA of synphilin-1 was subcloned into pGADT7 (a Gal4 DNA-activating domain vector for Gal4-AD fusion), and the cDNA of each mutant of NUB1 was subcloned into pGBKT7 (a Gal4 DNA-binding domain vector for Gal4-BD fusion). The plasmids of the two fusion constructs were then co-transfected into AH109 yeast cells using the lithium acetate method.[22] Transformed yeast cells were grown on a $His^-/Trp^-/Leu^-$ synthetic agar plate for 3 days at 30°C. The specific

protein-protein interaction was determined by the growth of the cells on the selection plate.

GST Pull-Down Assay

We performed a GST pull-down assay to confirm the result of the yeast two-hybrid interaction between synphilin-1 and NUB1. RH (RGSHHHHHH)-tagged synphilin-1 and GST fusion proteins, including GST, GST-HHR23B, and GST-NUB1, were expressed in *E. coli* BL21 cells by transformation with the pTrcHis plasmid and pGEX-2TK plasmid, respectively. Cells were then resuspended in the lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, and 0.1% Nonidet P-40) containing the protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and then lysed by brief sonication. The GST fusion proteins were purified using glutathione-Sepharose beads (Amersham Pharmacia Biotech) as described previously.[23] The bacterial crude lysate containing RH-synphilin-1 was centrifuged at 14,000 × *g* for 5 minutes, and the supernatant was incubated for 3 hours at room temperature with GST fusion proteins immobilized on glutathione-Sepharose beads. The beads were then washed four times with the lysis buffer. The precipitated RH-synphilin-1 on the beads was solubilized in 2% sodium dodecyl sulfate (SDS) treating solution (75 mmol/L Tris-HCl, pH 6.8, 2.0% SDS, 25% glycerol, 5% β-mercaptoethanol), followed by Western blot analysis using anti-RH antibody and anti-GST antibody.

Western Blot Analysis

Protein samples were treated for 1 hour at 50°C in 2% SDS treating solution. After SDS-polyacrylamide gel electrophoresis, Western blot analysis was performed according to the protocol provided with the ECL detection system (Amersham Pharmacia Biotech). Horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody (Santa Cruz Biotechnology) was used as a secondary antibody.

Northern Blot Analysis

Northern blotting was performed to show the mRNA expression of NUB1 in various human tissues. For this analysis, a human NUB1 cDNA fragment of 450 bp was amplified by PCR using pcDNA3/RH-NUB1 as the template[6,20] and subcloned into the pGEM-T plasmid (Promega, Madison, WI). The insert was then excised and labeled with [α-^{32}P]-dCTP by the Ready-To-Go DNA labeling kit (Amersham Pharmacia Biotech). The radioactive probe was then hybridized with two human multiple-tissue Northern blots (Clontech) in ExpressHyb solution (Clontech). After washing, the blot membrane was exposed to film for 5 days. As a control, the radioactive probe of β-actin was hybridized with the Northern blots, followed by exposure to film for 2 days.

Immunohistochemistry

Immunohistochemical studies were performed to determine the presence of NUB1 in samples of brains from patients with PD ($n = 5$), DLB ($n = 5$), and MSA ($n = 5$), as well as brain samples from normal subjects ($n = 5$), which were obtained from the Department of Pathology, Brain Research Institute, University of Niigata, Niigata, Japan, and the Department of Neuropathology, Hirosaki University School of Medicine, Hirosaki, Japan. Brains were fixed with 10% buffered formalin for 3 weeks and then embedded in paraffin. For this study, serial 4-μm-thick sections were prepared. We routinely deparaffinized and rehydrated sections from the midbrain and upper pons of cases of PD, the temporal lobe of cases of DLB, the upper pons of cases of MSA, and the temporal lobe and brainstem of normal controls. The sections were immunostained using the avidin-biotin-peroxidase complex method with diaminobenzidine as described previously.[11,24] The antibodies used for the immunostaining were polyclonal anti-NUB1 (2 μg/ml), polyclonal anti-synphilin-1 (1:500), and monoclonal anti-phosphorylated α-synuclein (1:5000). The sections were then counterstained with hematoxylin. The total number of inclusions immunostained with anti-NUB1 and anti-phosphorylated α-synuclein was quantified in contiguous sections.

Selected sections from the brainstem of PD, the temporal lobe of DLB, and the pons of MSA were double-immunolabeled with anti-NUB1 (10 μg/ml) and anti-phosphorylated α-synuclein antibodies (1:500). The secondary antibodies used were fluorescein isothiocyanate-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and Texas Red-conjugated anti-mouse IgG (Vector Laboratories). The sections were examined with an Olympus Provis fluorescence microscope (Olympus, Tokyo, Japan).

Immunocytochemistry

To investigate synphilin-1-positive inclusions in cultured cells, we performed immunocytochemical studies. HEK293 cells were cultured on a coverslip in a 3.5-cm dish and transfected with 2 μg of pNAC-Sph1. After 24 hours, the cells were fixed with a 4% paraformaldehyde solution, pH 7.5, for 30 minutes and permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature. The cells were first labeled with one of the following primary antibodies: mouse anti-FLAG (1:1800) for FLAG-NAC, rabbit anti-NUB1 (1:2000), mouse anti-Rpt5/PA700 (1:20,000), mouse anti-20S proteasome core subunits (1:4000),[25] mouse anti-ubiquitin (1:200), or rabbit anti-NEDD8 (1:2000). After washing, the cells were labeled with Texas Red-conjugated anti-mouse IgG (1:400) or anti-rabbit IgG (1:400) secondary antibody (Santa Cruz Biotechnology). The cells were then analyzed under a fluorescence microscope (Axioplan 2 Imaging; Carl Zeiss, Thornwood, NY). The localization of EGFP-fused synphilin-1 was shown by the green fluorescence of EGFP, and the localization of other proteins was shown by the red fluorescence of Texas Red. Their co-localization with synphilin-1 was shown by the merging of both fluorescences.

Quantification of Cells with Inclusions

HEK293 cells were cultured on a coverslip in a 3.5-cm dish and transfected with the following plasmids: 1) pEGFP (1 μg) and pcDNA3 (1 μg), 2) pNAC-EGFP (1 μg) and pcDNA3 (1 μg), 3) pSph1-EGFP (1 μg) and pcDNA3 (1 μg), 4) pNAC-Sph1 (1 μg) and pcDNA3 (1 μg), and 5) pNAC-Sph1 (1 μg) and pcDNA3/FLAG-NUB1 (1 μg). Twenty-four hours after transfection, the cells were fixed in a 4% paraformaldehyde solution, pH 7.5, and examined under a fluorescence microscope. Cells expressing EGFP or synphilin-1-EGFP were counted to determine the number of transfected cells. The transfected cells containing cytoplasmic inclusions were also counted. The value of percent cells with inclusions was calculated as the ratio of the number of transfected cells containing inclusions to the total number of transfected cells. All values were calculated from three independent experiments.

RNA Interference (RNAi)

To inhibit the expression of endogenous NUB1 in HEK293 cells, RNAi was used. Four short interfering RNAs (siRNAs) were designed and synthesized by Dharmacon (Lafayette, CO). The effect of these siRNAs was tested in our lab. Because one of the siRNAs showed complete inhibition of NUB1 expression, we used this siRNA for RNAi of NUB1. The NUB1 siRNA sequences, corresponding to nucleotides 458 to 476 after the start codon, were as follows: 5′-CGAUGGUGCUUGAACUAAAUU-3′ and 5′-UUUAGUUCAAGCACCAUCGUU-3′. The NUB1 siRNA or control siRNA was transfected for RNAi. Briefly, the siRNA and a plasmid DNA (pNAC-SphI) were co-transfected into HEK293 cells by Lipofectamine 2000 (Invitrogen).

Filter-Trap Assay of Inclusions

We performed a filter-trap assay to determine the experimental conditions under which inclusions were solubilized for the purpose of performing biochemical studies of inclusion formation. We modified the filter-trap assay method described previously.[26] In brief, cells were lysed in a lysis buffer [20 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, 0.01% SDS, protease inhibitor cocktail (Roche)] and sonicated for 10 seconds. The samples were boiled for 2 minutes and immediately applied to a 0.22-μm cellulose acetate membrane (Osmonics Inc., Minnetonka, MN) on a dot-blot apparatus (Millipore, Billerica, MA) using a vacuum manifold. After a 20-minute incubation at room temperature, the membrane was washed three times with a wash buffer (20 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, 0.08% SDS). The membrane was removed and Western blotted as described above.

TALON-Bead Precipitation of RH-Synphilin-1

To solubilize all derivatives of RH-synphilin-1 in 6 mol/L guanidine HCl and biochemically analyze them, we performed TALON-bead precipitation of RH-synphilin-1 as described previously.[27] Briefly, 1×10^6 HEK293 cells were co-transfected by FuGENE6 (Roche) to express RH-synphilin-1, HA-ubiquitin, NAC, and FLAG-NUB1. Twenty-four hours after transfection, the culture medium was replaced with fresh medium or medium containing 20 μmol/L MG132 (Calbiochem, San Diego, CA) and cultured at 37°C for 4 hours. The cells were then harvested and lysed in lysis buffer (20 mmol/L Tris-HCl, pH 8.0, 6 mol/L guanidine-HCl, 100 mmol/L NaCl). In this lysis buffer, all proteins, including deubiquitinating enzymes, were denatured by 6 mol/L guanidine HCl. Therefore, the ubiquitinated synphilin-1 was stable during the procedure. DNA in the lysate sample was sheared with a 22-gauge needle. The lysate was then incubated with cobalt-immobilized TALON beads (Clontech) for 1 hour at room temperature. Because the sequence of the RH tag was RGSHHHHHH, RH-synphilin-1 could be purified by TALON beads.[2,27] The beads were washed once with the lysis buffer and then washed twice with washing buffer (20 mmol/L Tris-HCl, pH 7.0, 15 mmol/L imidazole, 8 mol/L urea, 100 mmol/L NaCl). Finally, the beads were washed twice with phosphate-buffered saline and treated for 1 hour at 50°C in 2% SDS treating solution. The solubilized proteins were analyzed by Western blotting using anti-HA antibody and anti-RH antibody.

Statistical Analysis

All values were presented as means ± SE. Statistical significance of the data were evaluated using analysis of variance, followed by post hoc tests using the Fisher's adjustment or the Student's *t*-test when comparing two conditions. Probability values less than 0.05 ($P < 0.05$) were considered significant and probability values less than 0.01 ($P < 0.01$) were considered highly significant.

Results

NUB1 Interacts with Synphilin-1 in Yeast

We have previously identified the novel NEDD8-interacting protein NUB1. To investigate the molecular function of NUB1, we searched NUB1-interacting proteins by yeast two-hybrid screening using NUB1 as bait. Because the mRNA of NUB1 is highly enriched in the testis,[6] a human testis cDNA library was used for the screening. Approximately 2×10^6 primary library transformants were inoculated onto selection plates. A total of 84 colonies grew on the selection plates, 40 of which stained positive when tested for β-galactosidase expression. Subsequent DNA sequencing of the positive clones showed that two clones encoded synphilin-1. The rest of the positive clones included 2 clones of NEDD8,[6] 25 clones of UbC1,[20] and the other 11 clones that were not investigated yet.

NUB1 Directly Interacts with Synphilin-1

The result mentioned above indicated that synphilin-1 interacts with NUB1 directly or indirectly *in vivo*. To further investigate the interaction, an *in vitro* interaction assay was performed. RH-tagged synphilin-1 was expressed in

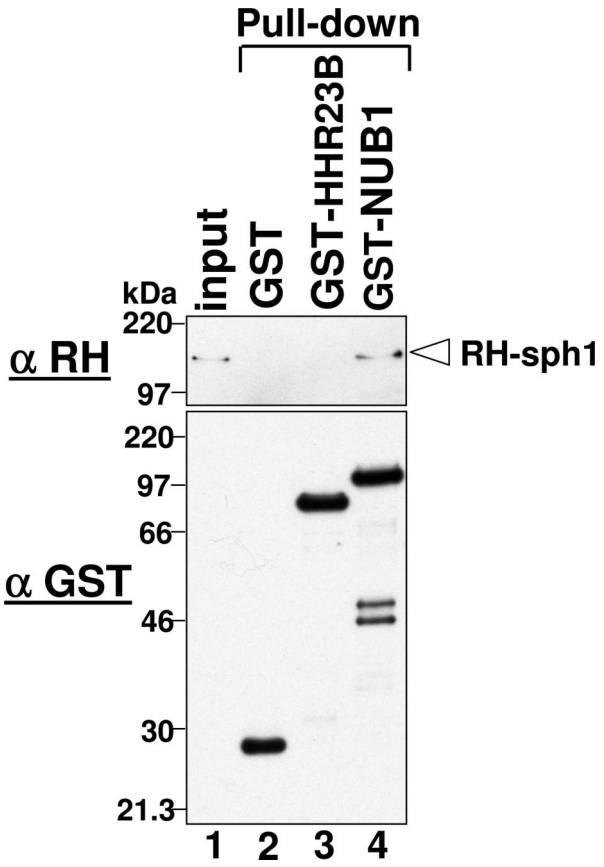


Figure 1. *In vitro* interaction between NUB1 and synphilin-1. GST and GST-fused HHR23B and NUB1 were expressed in bacteria and purified using glutathione-Sepharose beads. RH-tagged synphilin-1 (sph1) expressed in bacteria was then precipitated with these beads, which were coated with GST alone (**lane 2**), GST-HHR23B (**lane 3**), or GST-NUB1 (**lane 4**). The precipitates were analyzed by Western blotting using anti-RH antibody to detect RH-synphilin-1 (**top**) and anti-GST antibody to detect immobilized GST, GST-HHR23B, or GST-NUB1 (**bottom**). In **lane 1**, the input of RH-synphilin-1 diluted to 1:40 was loaded as a control. Molecular size markers are shown in kilodaltons.

bacteria. The bacterial lysate containing RH-synphilin-1 was then incubated with GST alone (negative control), GST-fused HHR23B (negative control), or GST-fused NUB1 and precipitated by the GST pull-down method. The precipitate was then analyzed by Western blotting using anti-RH antibody. As shown in Figure 1, top, RH-synphilin-1 was precipitated with the GST-NUB1 (Figure 1, lane 4), but not with either GST alone (Figure 1, lane 2) or GST-HHR23B (Figure 1, lane 3). In this assay, the input diluted to 1:40 was loaded as a control (Figure 1, lane 1). The detected level of RH-synphilin-1 in Figure 1, lane 4, is almost equal to that in Figure 1, lane 1, suggesting that ~2.5% of RH-synphilin-1 was precipitated by GST-NUB1. Taken together, these results indicated that synphilin-1 directly and specifically interacted with NUB1.

NUB1 Interacts with Synphilin-1 Through Its NEDD8-Binding Site

We precisely identified the synphilin-1-binding site on NUB1 using deletion mutants of NUB1 in a yeast two-hybrid interaction assay. As shown in Figure 2A, we generated eight mutants of NUB1, m1 to m8, to examine the interaction with synphilin-1. Each mutant has a C-terminal deletion and/or an N-terminal deletion. For example, m1 has a C-terminal deletion from Lys-371 to Asn-601, resulting in the loss of two UBA domains (UBA1 and UBA3) and a PEST domain. m2 has an N-terminal deletion from Met-1 to Phe-370, resulting in the loss of a UBL domain. Using these mutants and a wild-type NUB1, we then examined the interaction with synphilin-1 in yeast cells. In the yeast two-hybrid assay, synphilin-1 fused to the Gal4 activation domain was used for the interaction with a panel of NUB1 mutants fused to the Gal4 DNA-binding domain. As shown in Figure 2, A and B, synphilin-1 interacted with wild-type NUB1 (wt), $NUB1^{(371\text{-}601)}$ (m2), $NUB1^{(461\text{-}601)}$ (m6), and $NUB1^{(515\text{-}601)}$ (m8), but not with $NUB1^{(1\text{-}370)}$ (m1), $NUB1^{(1\text{-}418)}$ (m3), $NUB1^{(371\text{-}418)}$ (m4), $NUB1^{(427\text{-}460)}$ (m5), and $NUB1^{(472\text{-}514)}$ (m7). These results indicated that a synphilin-1-binding site was located at the C-terminus of NUB1 between amino acid residues 515 and 601. To pinpoint the synphilin-1-binding site at the C-terminus of NUB1, we generated five more mutants, m9 to m13, and examined their interaction with synphilin-1 using the yeast two-hybrid assay. As shown in Figure 2, C and D, synphilin-1 interacted with $NUB1^{(515\text{-}584)}$ (m9), $NUB1^{(536\text{-}601)}$ (m11), and $NUB1^{(536\text{-}584)}$ (m13), but not with $NUB1^{(515\text{-}568)}$ (m10) and $NUB1^{(569\text{-}601)}$ (m12). These results indicated that the synphilin-1-binding site was located at the C-terminus of NUB1 between amino acid residues 536 and 584. Importantly, this region was identical to the NEDD8-binding site[21] (Figure 2E).

NUB1 Is Expressed in the Human Brain

Because synphilin-1 physically interacts with NUB1 and is predominantly expressed in the brain,[8,28] NUB1 should also be expressed in the brain. However, our previous result of Northern blotting showed that 3.5 kb of NUB1 mRNA was weakly detected in some tissues but almost undetectable in the brain and that 2.3 kb of NUB1 mRNA was strongly detected in the testis but not in the other tissues.[6] To investigate whether the mRNA of NUB1 is expressed in the brain, we purchased the same human multiple tissue Northern blots and hybridized with the radioactive probe of NUB1. To increase the sensitivity, we exposed the blot membranes to a film longer. As shown in Figure 3A (top), 3.5 kb of NUB1 mRNA was weakly detected in the brain. We also performed Western blot analysis to determine the protein expression of endogenous NUB1 in various human tissues. As shown in Figure 3B, the 69-kd of NUB1 protein was predominantly expressed in the brain (Figure 3B, top). NUB1 was also expressed in the heart, kidney, and testis (Figure 3B, lanes 1, 3, and 6). In the testis, a double band was detected (Figure 3B, lane 6). The upper band might represent a gene product of the alternative splicing, which was detected in Northern blotting (Figure 3A). In the lung and spleen, the endogenous NUB1 was almost undetectable (Figure 3B, lanes 2 and 4). Interestingly, this expression level of NUB1 in various tissues was similar to that of synphilin-1 (Figure 3B, middle). Thus, we detected both the mRNA and protein of NUB1 in the human brain.

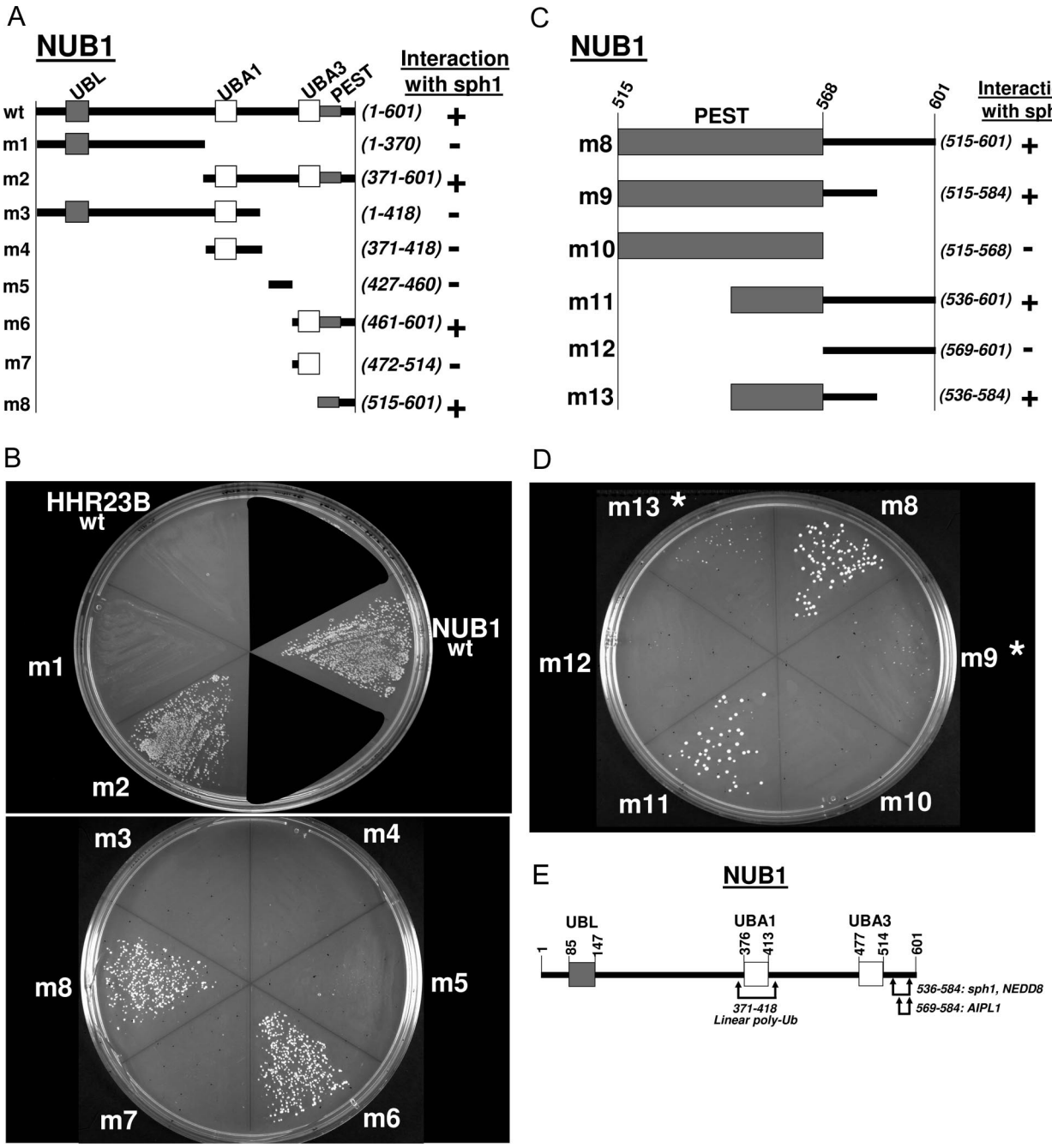


Figure 2. Mapping for the synphilin-1-binding site on NUB1 using a yeast two-hybrid system. **A:** Summary of interaction between synphilin-1 and truncated NUB1 (m1 to m8). **B:** Primary data of interaction between synphilin-1 and truncated NUB1 (m1 to m8). The yeast strain AH109 was transformed with pGADT7/synphilin-1 and the pGBKT7 construct encoding wild-type NUB1 (wt), truncated NUB1 (m1 to m8), or HHR23B as a negative control. Transformed yeast cells were grown on a selection plate to determine the specific protein-protein interaction. **C:** Summary of interaction between synphilin-1 and C-terminal mutant NUB1 (m8 to m13). **D:** Primary data of interaction between synphilin-1 and C-terminal mutant NUB1 (m8 to m13). The yeast strain AH109 was transformed with pGADT7/synphilin-1 and the pGBKT7 construct encoding truncated NUB1 (m8 to m13). Transformed yeast cells were grown on a selection plate to determine the specific protein-protein interaction. **Asterisks** indicate low growth of yeast colonies. **E:** Location of synphilin-1-binding site on NUB1, indicated by **arrows**. Binding sites of other proteins, such as NEDD8,[21] linear polyubiquitin,[20] and AIPL1,[43] are also indicated by **arrows**.

NUB1, as Well as Synphilin-1 and α-Synuclein, Is Accumulated in Inclusions of α-Synucleinopathies

α-Synuclein and synphilin-1 are major components of LBs found in the brains of patients with PD and DLB, as well as of glial cytoplasmic inclusions (GCIs) seen in the brains of patients with MSA.[11,29,30] Because NUB1 interacts with synphilin-1, we hypothesized that NUB1 is also present in the inclusion bodies in the brains of patients with α-synucleinopathies. To determine this, immunohistochemical investigations (Figure 4A) were performed on normal brains (Figure 4A, a–i) and the brains from patients with PD (Figure 4A, j, m, and p), DLB (Figure 4A, k,

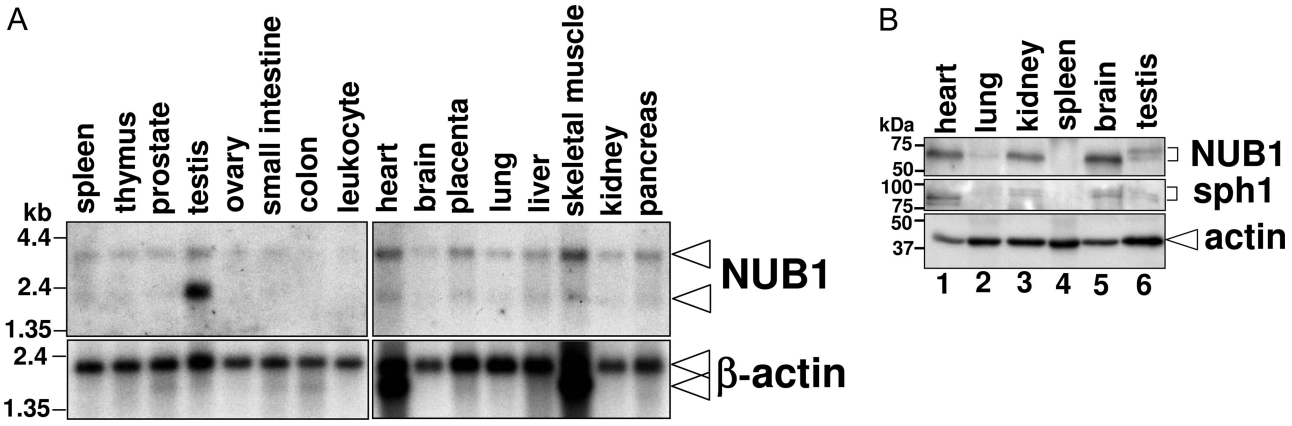


Figure 3. Expression of NUB1 in various human tissues. **A:** Northern blot analysis. mRNA expression of NUB1 (**top**) and β-actin (**bottom**) was examined in a variety of normal human tissues. RNA size markers are shown in kilobases. **B:** Western blot analysis. Protein expression of NUB1 (**top**), synphilin-1 (**middle**), and actin (**bottom**) was examined using a variety of human tissues purchased from ProSci (Poway, CA). Molecular size markers are shown in kilodaltons.

n, and q), or MSA (Figure 4A, l, o, and r) using control serum (Figure 4A, a–c and j–l), anti-NUB1 antibody (Figure 4A, d–f and m–o), and anti-synphilin-1 antibody (Figure 4A, g–i and p–r). As shown in Figure 4A, d–f, the anti-NUB1 antibody weakly immunostained the neuronal cytoplasm and processes in the normal brains. In the brains of patients with α-synucleinopathies, brainstem-type LBs (Figure 4Am), cortical LBs (Figure 4An), and GCIs (Figure 4Ao) were positive for NUB1. These findings together showed that NUB1, as well as synphilin-1 (Figure 4A, p–r), is present in LBs of PD and DLB and in GCIs of MSA.

Because α-synuclein is a major component in inclusions of α-synucleinopathies, we examined the relationship between α-synuclein and NUB1. As shown in Figure 4B, double-labeling immunofluorescence revealed co-localization of NUB1 and phosphorylated α-synuclein in inclusions of α-synucleinopathies. Furthermore, contiguous sections stained with anti-NUB1 and anti-phosphorylated α-synuclein revealed that 95% of brainstem-type LBs and 82% of cortical LBs were positive for NUB1. In MSA, 98% of GCIs were positive for NUB1. Thus, the vast majority of inclusions in human α-synucleinopathies contained NUB1.

Endogenous NUB1 and LB Components Localize in Synphilin-1-Positive Inclusions of HEK293 Cells

In HEK293 cells, cytoplasmic inclusions are formed when synphilin-1 is co-expressed with α-synuclein.[31] Interestingly, the inclusion formation is facilitated when synphilin-1 is co-expressed with the NAC portion (non-Aβ component of Alzheimer's disease amyloid) of α-synuclein.[8] In PD, it is possible that the proteolysis of α-synuclein, leading to a NAC-related polypeptide, facilitates LB formation. Indeed, various truncated forms of α-synuclein have been detected in LBs,[29,32,33] suggesting that the proteolytic processing of α-synuclein plays a role in LB formation. To experimentally examine the formation of cytoplasmic inclusions in cultured cells, we first established an inclusion-formation assay. As shown in Figure 5A, when we co-expressed EGFP-fused synphilin-1 (sph1-EGFP) with FLAG-tagged NAC, this led to the obvious formation of synphilin-1-containing inclusions resembling LBs (Figure 5Aa). The immunostaining of the cells further revealed that NAC was localized in the core of synphilin-1-positive inclusions (Figure 5A, b and c). These observations were consistent with those reported previously.[8,31] Thus, we established our own assay system for inclusion formation by co-expressing synphilin-1-EGFP with the NAC portion of α-synuclein in HEK293 cells.

Although NUB1 was detected in LBs of patient brains (Figure 4A), we did not know whether endogenous NUB1 was present along with synphilin-1 in the inclusions of cultured cells shown in Figure 5A. To investigate this possibility, HEK293 cells expressing synphilin-1-EGFP and FLAG-NAC were immunostained with anti-NUB1 antibody. As shown in Figure 5B, endogenous NUB1 was present along with synphilin-1 in the cytoplasmic inclusions (Figure 5B, a–c), probably because of the interaction between NUB1 and synphilin-1. In addition to NUB1, we examined the co-localization of synphilin-1 with other LB components, such as the 26S proteasome (consisting of PA700 and 20S core), ubiquitin, and NEDD8. As expected, synphilin-1 co-localized with the Rpt5 subunit of PA700 (Figure 5B, d–f), α-subunits of the 20S core (Figure 5B, g–i), ubiquitin (Figure 5B, j–l), and NEDD8 (Figure 5B, m–o). Such results show that the inclusion bodies in cultured cells are very similar to LBs in patient brains, indicating that our inclusion-formation assay system is a good tool for studying the role of NUB1 in either the formation or breakdown of inclusions.

NUB1 Down-Regulates the Formation of Synphilin-1-Positive Inclusions in HEK293 Cells

To determine whether NUB1 is involved in the formation or breakdown of synphilin-1-positive inclusions, we overexpressed RH-tagged NUB1 in HEK293 cells. Because inclusions were formed in cells co-expressing

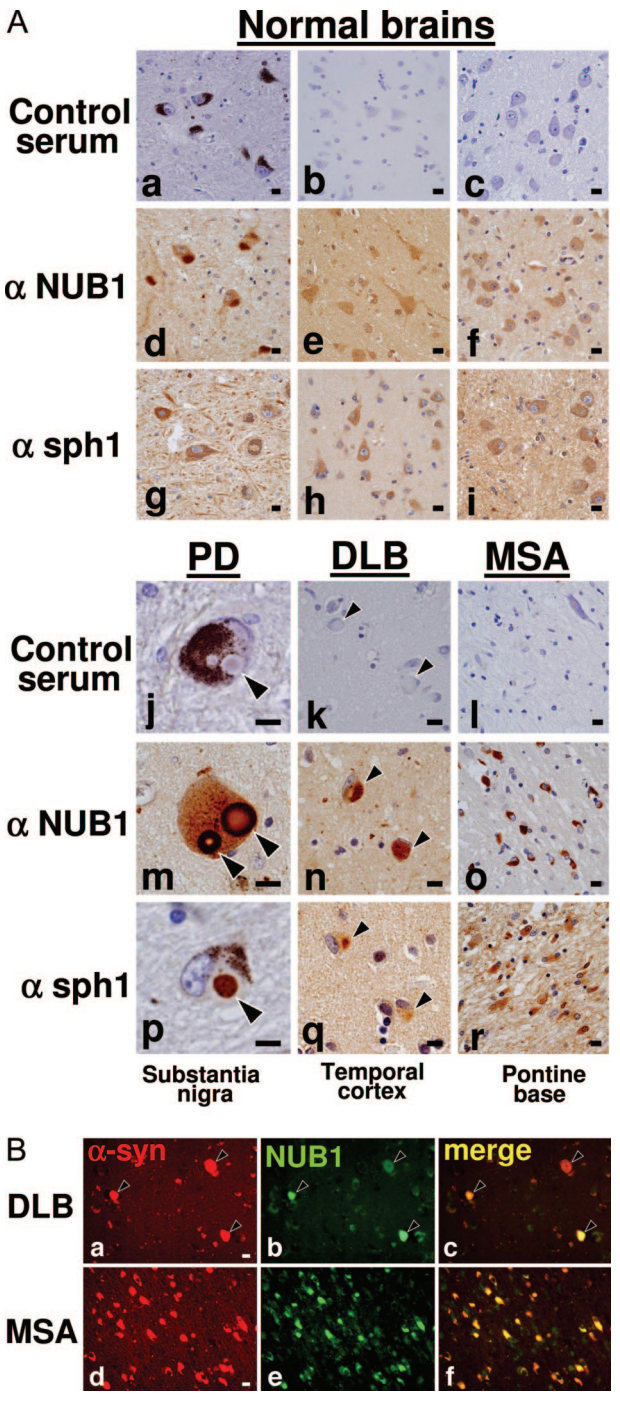


Figure 4. Localization of NUB1 in inclusions of patient brains. **A:** Localization of NUB1 and synphilin-1 in inclusion bodies of the brains of patients with neurodegenerative α-synucleinopathies. Immunohistochemical studies were performed on the substantia nigra (**a**, **d**, and **g**), temporal cortex (**b**, **e**, and **h**), and pontine base (**c**, **f**, and **i**) from control subjects and the substantia nigra from PD (**j**, **m**, and **p**), temporal cortex from DLB (**k**, **n**, and **q**), and pontine base from MSA (**l**, **o**, and **r**) using control serum (**a–c** and **j–l**), anti-NUB1 antibody (**d–f** and **m–o**), and anti-synphilin-1 antibody (**g–i** and **p–r**). Anti-NUB1 (**d–f**) and anti-synphilin-1 antibodies (**g–i**) weakly immunolabeled the neuronal cytoplasm and processes in the normal brains. LBs in the brains of patients with PD and DLB were immunostained with anti-NUB1 (**m** and **n**), as were glial cytoplasmic inclusions (GCIs) in patients with MSA (**o**). LBs and GCIs were also positive for synphilin-1 (**p–r**). **B:** Double-immunofluorescence staining showing co-localization of phosphorylated α-synuclein and NUB1 in cortical LBs in patients with DLB (**a–c**) and GCIs in patients with MSA (**d–f**). α-Synuclein appears red (**a** and **d**) and NUB1 appears green (**b** and **e**). The overlap of α-synuclein with NUB1 appears yellow (**c** and **f**). Scale bars = 10 μm.

synphilin-1-EGFP and NAC, we estimated the effect of the overexpression of RH-NUB1 on inclusion formation using this assay system. As shown in Figure 6A, inclusions were not generated when EGFP alone (Figure 6Aa) or EGFP plus NAC (Figure 6Ab) were expressed. In contrast, inclusions were generated when synphilin-1-EGFP (Figure 6A, c–e) was expressed. Specifically, when synphilin-1-EGFP was expressed alone, 2.2% of cells generated inclusions (Figure 6Ac). When synphilin-1-EGFP and NAC were expressed, 11.8% of cells generated inclusions (Figure 6Ad). Importantly, when synphilin-1-EGFP and NAC were co-expressed with RH-NUB1, the number of inclusion-positive cells was reduced to 5.4% (Figure 6Ae). This result suggests that NUB1 down-regulates the formation of synphilin-1-positive inclusions in HEK293 cells.

Next, we determined the location of synphilin-1-EGFP and RH-NUB1 in HEK293 cells shown in Figure 6Ae. As shown in Figure 6B, synphilin-1-EGFP was mainly located in the inclusions, and was faintly present in the cytoplasm. The overexpressed RH-NUB1 was located in the nucleus, cytoplasm, and inclusions. Importantly, RH-NUB1 co-localized with synphilin-1-EGFP to both the cytoplasm and the periphery of the synphilin-1-positive inclusions.

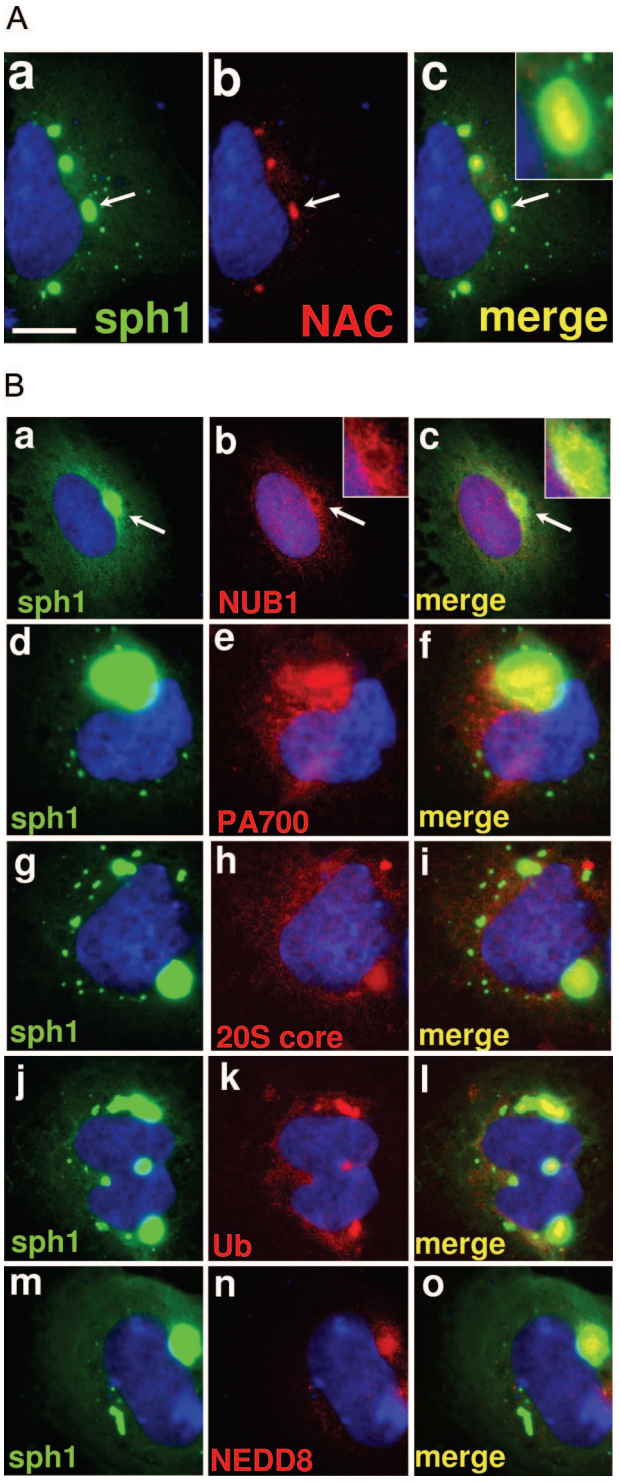
RNAi of NUB1 Does Not Cause Any Effects on the Formation of Synphilin-1-Positive Inclusions in HEK293 Cells

We knocked down the endogenous NUB1 by transfecting siRNA. As shown in Figure 7A, the siRNA of NUB1 completely inhibited the expression of endogenous NUB1 in HEK293 cells (Figure 7A, lane 2 versus lane 1). Using this system, we further investigated the role of NUB1 in the formation of synphilin-1-positive inclusions in HEK293 cells. As shown in Figure 7B, the transfection with NUB1 siRNA did not cause any effects on the formation of synphilin-1-positive inclusions in HEK293 cells (Figure 7B, lane 2 versus lane 1). This is probably because other proteins compensate for the function of NUB1 in HEK293 cells.

Synphilin-1-Positive Inclusions in HEK293 Cells Are Solubilized in 0.1% SDS, 8 mol/L Urea, or 3 mol/L Guanidine Solution

To biochemically investigate the role of NUB1 in inclusion formation, we first established the method to prepare total cell lysate. As described above, the expression of synphilin-1 led to the inclusion formation. For biochemical studies of the inclusion formation, we had to determine the experimental condition under which inclusions were solubilized. For this purpose, a filter trap-assay[26] was performed. As shown in Figure 8A, the RH-synphilin-1-containing inclusions were efficiently trapped on a 0.22-μm cellulose acetate filter (sample 2). When NAC was co-expressed in HEK293 cells, we detected more inclusions (sample 2 versus sample 3). This result was consistent with that from our morphological study (Figure 6A, sample c versus sample d). Using this assay system,

we next examined the solubility of RH-synphilin-1-containing inclusions in various solutions. As shown in Figure 8B, inclusions were solubilized in 0.1% SDS, 8 mol/L urea, 3 mol/L guanidine HCl, or 6 mol/L guanidine HCl. The solubilized RH-synphilin-1 could not be trapped on the filter membrane. In contrast, the inclusions were not completely solubilized in 0.01% SDS, 4 mol/L urea, or 1.5 mol/L guanidine HCl, because the inclusions were trapped on the filter membrane.



NUB1 Down-Regulates the Formation of Synphilin-1-Positive Inclusions Through the Proteasomal Degradation of Synphilin-1

The role of NUB1 in the formation or breakdown of synphilin-1-positive inclusions was biochemically investigated. Specifically, we co-expressed RH-synphilin-1, HA-ubiquitin, and NAC with different amounts of FLAG-NUB1 in HEK293 cells (Figure 8C, top). The transfected cells were then cultured in the presence or absence of the proteasome inhibitor MG132. After the incubation, the cells were harvested. Based on the results of the filter-trap assay, we then prepared the total cell lysate using 6 mol/L guanidine HCl, which allowed us to denature and solubilize all derivatives of RH-synphilin-1, including those in the inclusions (Figure 8B). Afterward, the derivatives of RH-synphilin-1 were precipitated with cobalt-coated TALON beads. The precipitates were then analyzed by Western blotting (Figure 8C) using anti-HA antibody to detect the ubiquitinated form of RH-synphilin-1 (Figure 8C, middle) and anti-RH antibody to detect RH-synphilin-1 (Figure 8C, bottom).

As shown in Figure 8C, when RH-synphilin-1 was expressed with HA-ubiquitin and NAC in HEK293 cells without the co-expression of FLAG-NUB1 (Figure 8C, lane 1), RH-synphilin-1 was strongly detected (Figure 8C, bottom). In addition, the ubiquitinated RH-synphilin-1 was clearly detected (Figure 8C, middle). When FLAG-NUB1 was co-expressed, however, the expression of RH-synphilin-1 and its ubiquitinated form was decreased commensurate with the level of FLAG-NUB1 expression (Figure 8C, lanes 2 to 4). Importantly, a proteasome inhibitor, MG132, completely blocked this reduction of RH-synphilin-1 and its ubiquitinated form (Figure 8C, lanes 5 to 8).

As described above, the polyubiquitination of synphilin-1 was detected in this study by using anti-HA antibody and anti-RH antibody. The polyubiquitination was clearly detected by anti-HA antibody (Figure 8C, middle), whereas it was almost undetectable by anti-RH antibody (Figure 8C, bottom). Why did the use of these two antibodies produce such discrepant results in the detection of polyubiquitination? A polyubiquitin chain on RH-syn-

Figure 5. Localization of NUB1 in inclusions of cultured HEK293 cells. **A:** Synphilin-1-positive inclusions in HEK293 cells transfected with synphilin-1 and α-synuclein. HEK293 cells were transfected with pNAC-Sph1 to express both FLAG-NAC and synphilin-1-EGFP. After 24 hours, the cells were fixed and immunostained with anti-FLAG antibody. The primary antibody was then labeled with Texas Red-conjugated secondary antibody. The immunostained cells were treated with Hoechst 33258 dye for the nuclear staining and then analyzed under a fluorescence microscope. The localization of synphilin-1-EGFP was shown by the green fluorescence of EGFP (**a**), and the localization of FLAG-NAC was shown by the red fluorescence of Texas Red (**b**). Their co-localization was shown by the merging of both fluorescences (**c**). **B:** Co-localization of synphilin-1 with endogenous proteins related to LBs in inclusions of HEK293 cells. HEK293 cells were transfected with pNAC-Sph1. After 24 hours, the cells were fixed and immunostained with antibodies to NUB1 (**a–c**), the Rpt5 subunit of PA700 (**d–f**), the α-subunits of 20S proteasome (**g–i**), ubiquitin (Ub; **j–l**), or NEDD8 (**m–o**). The primary antibody was then labeled with Texas Red-conjugated secondary antibody. The immunostained cells were treated with Hoechst 33258 dye for the nuclear staining and then analyzed under a fluorescence microscope. The localization of synphilin-1-EGFP was shown by the green fluorescence of EGFP (**a**, **d**, **g**, **j**, and **m**), and the localization of other endogenous proteins was shown by the red fluorescence of Texas Red (**b**, **e**, **h**, **k**, and **n**). Their co-localization with synphilin-1 was shown by the merging of both fluorescences (**c**, **f**, **i**, **l**, and **o**). Scale bar = 10 μm.

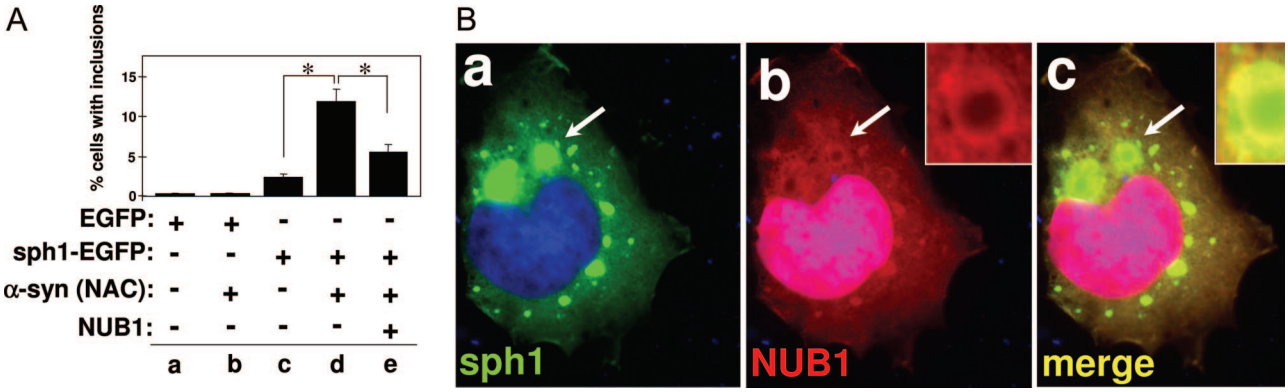


Figure 6. NUB1-mediated regulation in the formation of synphilin-1-positive inclusions. **A:** Effect of NUB1 overexpression on the formation of synphilin-1-positive inclusions. In HEK293 cells transfected with various constructs, cytoplasmic inclusions with green fluorescence were quantified. Cells expressing EGFP (**a** and **b**) or synphilin-1-EGFP (**c–e**) were counted to determine the number of transfected cells. Then, the transfected cells containing cytoplasmic inclusions were counted. The value of percent cells with inclusions was calculated as the ratio of the number of transfected cells containing inclusions to the total number of transfected cells. Each bar represents the mean ± SE (*$P < 0.0001$). **B:** Location of overexpressed NUB1 in synphilin-1-positive inclusions. As done in **Ae**, RH-NUB1 was co-expressed with synphilin-1-EGFP and NAC in HEK293 cells. After 24 hours, the cells were fixed and immunostained with anti-RH antibody (1:1600). The primary antibody was then labeled with Texas Red-conjugated secondary antibody (1:400). The immunostained cells were treated with Hoechst 33258 dye for the nuclear staining and then analyzed under a fluorescence microscope. The location of synphilin-1-EGFP was shown by the green fluorescence of EGFP (**a**), and the location of RH-NUB1 was shown by the red fluorescence of Texas Red (**b**). Their co-localization was shown by the merging of both fluorescences (**c**).

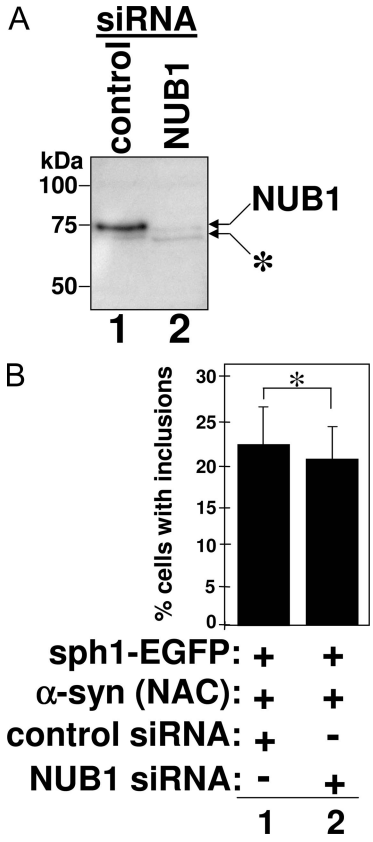


Figure 7. RNAi of NUB1. **A:** Effect of NUB1 siRNA on the expression of endogenous NUB1. HEK293 cells were transfected with a siRNA of control or NUB1 and a plasmid encoding synphilin-1-EGFP and FLAG-NAC. Twenty-four hours after transfection, cells were lysed. The expression level of endogenous NUB1 was then determined by Western blotting using anti-NUB1 antibody. A nonspecific band is indicated by an **asterisk**. **B:** Effect of NUB1 siRNA on the formation of synphilin-1-positive inclusions. HEK293 cells were transfected with a siRNA of control or NUB1 and a plasmid encoding both synphilin-1-EGFP and FLAG-NAC. Twenty-four hours after transfection, cells were fixed, and then the transfected cells containing cytoplasmic inclusions were counted under a fluorescence microscope. Each bar represents the mean ± SE (*$P = 0.532$, not significant).

philin-1 consists of multiple molecules of HA-ubiquitin. Because each of these molecules reacts with an anti-HA antibody, the polyubiquitin chain is labeled with multiple molecules of anti-HA antibody. This is the reason why anti-HA antibody detects the polyubiquitin chain much more strongly than actual. In contrast, detection of the polyubiquitinated RH-synphilin-1 by anti-RH antibody reflects the actual level of the expression because the anti-RH antibody reacts only with a single RH-epitope of the polyubiquitinated RH-synphilin-1.

Discussion

Synphilin-1 was initially identified as an α-synuclein-interacting protein. In normal or physiological condition, synphilin-1 is predominantly expressed in neurons, located in the cytoplasm and presynaptic nerve terminals, and associated with synaptic vesicles.[8,28] However, in several neurodegenerative disorders called α-synucleinopathies, such as PD, DLB, and MSA, synphilin-1 is mainly localized in neuronal and glial cytoplasmic inclusions,[10,11] in which α-synuclein,[32,34] ubiquitin,[35] NEDD8,[14,36] and the proteasome[25,37] are also present. Experimental studies have revealed that the co-expression of synphilin-1 with α-synuclein results in the formation of LB-like cytoplasmic inclusions in cultured HEK293 cells.[8,31] Furthermore, it is of interest that a novel Arg621Cys mutation has been identified in the synphilin-1 gene in two sporadic PD patients.[18] These observations indicate that synphilin-1 is involved in the formation of pathological inclusions and also in the pathogenesis of several neurodegenerative α-synucleinopathies.

In the present study, we showed that NUB1, a synphilin-1-interacting protein, is expressed in the normal human brain. Using an immunohistochemical staining method, we further showed that NUB1, as well as synphilin-1, is highly accumulated in the inclusion bodies of the brains of patients with neurodegenerative α-synucle-

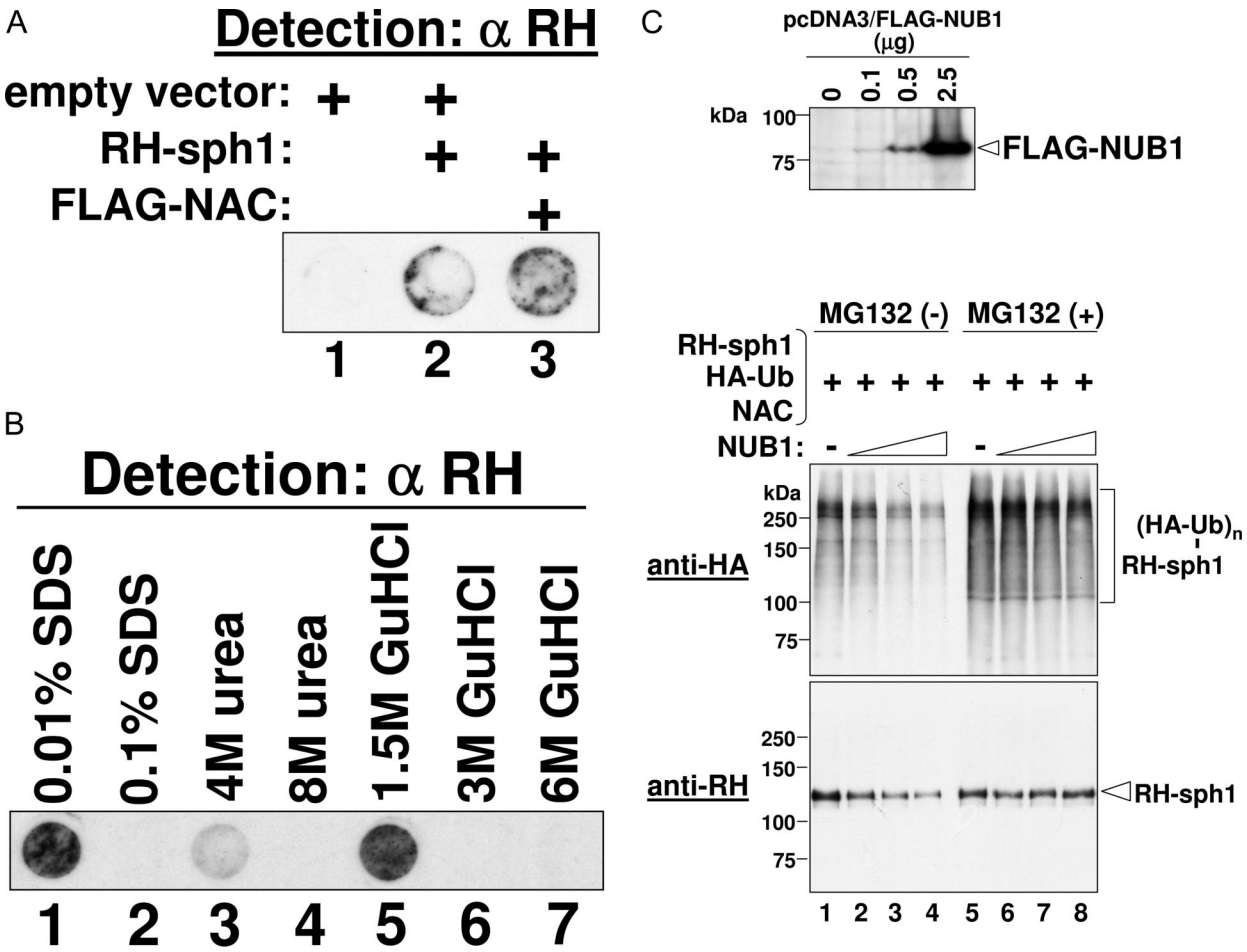


Figure 8. Reduction of synphilin-1 by overexpression of NUB1 in HEK293 cells. **A:** Filter-trap assay of synphilin-1-positive inclusions. The inclusions were generated by overexpressing RH-synphilin-1 with or without NAC in HEK293 cells. The inclusions were then trapped on a 0.22-μm cellulose acetate membrane and detected with anti-RH antibody. **B:** Solubilization of synphilin-1-positive inclusions in various solutions. The inclusions were generated by overexpressing RH-synphilin-1 with NAC in HEK293 cells. Total cell lysate was prepared from the cells in various solutions and analyzed by filter-trap assay. **C:** NUB1-mediated proteasomal degradation of synphilin-1. By transfecting plasmids, FLAG-NUB1 was overexpressed at various levels with RH-synphilin-1, HA-ubiquitin, and NAC in HEK293 cells. A portion of the cells were lysed for Western blot analysis using anti-FLAG antibody to detect FLAG-NUB1 (**top**). The rest of the cells were further cultured in the presence or absence of MG132. The cells were then lysed in 6 mol/L of guanidine HCl, and a total cell lysate was prepared. RH-synphilin-1 was precipitated with TALON beads from the total cell lysate and analyzed by Western blotting using anti-HA antibody to detect ubiquitinated RH-synphilin-1 and anti-RH antibody to detect RH-synphilin-1 (**middle** and **bottom**). Note: In all samples, 2.5 μg of plasmid DNA for the expression of RH-synphilin-1, HA-ubiquitin, and NAC were transfected into HEK293 cells on a 6-cm dish. In addition, the cells were co-transfected with a plasmid for the expression of FLAG-NUB1 in amounts of 0 μg (**lanes 1** and **5**), 0.1 μg (**lanes 2** and **6**), 0.5 μg (**lanes 3** and **7**), or 2.5 μg (**lanes 4** and **8**).

inopathies, such as PD, DLB, and MSA. In this study, however, NUB1 could not be detected in 5% of brainstem-type LBs and 18% of cortical LBs. It might result from the low sensitivity of the anti-NUB1 antibody used for the immunostaining. Although we need to generate more sensitive anti-NUB1 antibodies to clarify the exact extent of NUB1 deposition in LBs, our immunostaining results suggest that NUB1 plays a role in the formation or breakdown of inclusions through its interaction with synphilin-1. To test this possibility, we first established an assay system in which we experimentally generated synphilin-1-positive inclusions by co-expressing synphilin-1 with the NAC portion of α-synuclein in HEK293 cells. Previously, the experimental system similar to ours was characterized well.[38] In the study, O'Farrell and colleagues[38] demonstrated that overexpression of synphilin-1 forms cytoplasmic inclusions in HEK293 cells. However, they found that the nature of these inclusions is different from LBs *in vivo* because these inclusions are not shown to be filamentous and instead are membrane bound.[38] Despite these differences, we still performed our inclusion formation assay using HEK293 cells to get insights into the mechanism of LB formation and to test our hypothesis, because there is no alternative method.

Recently, synphilin-1 was shown to be ubiquitinated by four RING-finger-containing ubiquitin E3 ligases, parkin, siah-1 and -2, and dorfin.[8,25,39,40] The functional similarity of these E3 ligases indicates that multiple pathways are facilitating the ubiquitination of synphilin-1. Interestingly, siah proteins ubiquitinate synphilin-1, promoting its degradation by the ubiquitin-proteasome system.[39,40] Unlike siah proteins, parkin assembles a lysine 63-linked polyubiquitin chain on synphilin-1 that is distinct from the classical, degradation-associated, lysine 48-linked ubiquitination.[41,42] So far, it has been unknown which type of polyubiquitin chain is assembled on synphilin-1 by dorfin.[25] In the past, some groups overexpressed these E3 ligases in HEK293 cells to promote the formation of in-

clusions.[31,40] However, it is still unclear how the overexpression of these E3 ligases plays a role in the formation of inclusions.[39–42] In our inclusion-formation assay, these E3 ligases were not overexpressed, because we could efficiently generate synphilin-1-positive inclusions without the overexpression of E3 ligases. In this way we were able to investigate the role of NUB1 in the formation of inclusions under the physiological expression of E3 ligases. Using this inclusion-formation assay, we clearly showed that NUB1 and other LB components, including ubiquitin, NEDD8, and the proteasome, are localized in synphilin-1-positive inclusions, suggesting that inclusions generated in our assay are similar to LBs in patient brains. Furthermore, our assay revealed that NUB1 down-regulated the formation of synphilin-1-positive inclusions. The question now is: how does NUB1 down-regulate the formation of inclusions? As described below, the involvement of NUB1 in the NEDD8-proteasome pathway gave us a clue to the molecular mechanism responsible for this down-regulation.

Previously, we demonstrated that NUB1 directly interacts with a ubiquitin-like protein NEDD8 through its C-terminal region and targets NEDD8 to the proteasome for degradation.[7,21] This showed that NUB1 functions as a molecular chaperone for NEDD8 to be targeted to the proteasome. Because we found that NUB1 interacts with synphilin-1 through its NEDD8-binding site, we hypothesized that NUB1 not only targets NEDD8 but also synphilin-1 to the proteasome for degradation. Indeed, in the present study, we found that NUB1 overexpression led to a reduction in the expression of synphilin-1. Most importantly, this reduction was completely blocked by the proteasome inhibitor MG132, suggesting that NUB1 reduces the expression of synphilin-1 by means of proteasomal degradation. Thus, these results strongly support our hypothesis described above. Furthermore, these results also help explain the molecular mechanism by which NUB1 down-regulates the formation of synphilin-1-positive inclusions in HEK293 cells. That is, the NUB1-mediated targeting of synphilin-1 to the proteasome causes the efficient degradation, and hence reduction, of synphilin-1, thereby suppressing the formation of synphilin-1-positive inclusions.

Although we defined that NUB1 promotes the proteasomal degradation of synphilin-1 in cultured cells, the function of NUB1 in human brain has not been elucidated. However, we believe that NUB1 plays the same role in cells of human brain. Because both NUB1 and synphilin-1 are expressed in the normal brain, NUB1 should also promote the proteasomal degradation of synphilin-1 in the cells of normal brain through its interaction with synphilin-1. In the brains of patients with α-synucleinopathies, such as PD, DLB, and MSA, NUB1 should also reduce the expression of synphilin-1 and thereby down-regulate the formation of synphilin-1-positive inclusions. Thus, NUB1 seems to play an important role in brain cells under both physiological and pathological conditions. Currently, we are generating *nub1*-knockout mice, which will further define the function of NUB1 in the brain.

In addition to the basic science aspects, our findings on NUB1 have two important bearings clinically. First, they suggest that NUB1 could serve as a neuropathological marker in patients with α-synucleinopathies because it is strongly accumulated with synphilin-1 in the inclusions of their brain cells. Second, they suggest that NUB1 could be a potential therapeutic target for α-synucleinopathies.

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